Leukocyte adhesion: Two selectins converge on sulphate

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Selectins mediate the attachment of leukocytes to endothelial cells and to platelets under conditions of blood flow. Sulphation of the ligands for two of the selectins is required for optimal cell–cell interactions.

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The selectins are a family of cell–cell adhesion proteins that participate in leukocyte–endothelial and leukocyte–platelet interactions [1–4]. The family consists of three members: P-selectin, expressed on activated endothelium and platelets; E-selectin, expressed on activated endothelium; and L-selectin, expressed on circulating leukocytes (Fig. 1). During the process of leukocyte emigration into inflammatory sites or lymphoid organs, the selectins are thought to initiate and sustain the rolling of blood-borne leukocytes on the endothelial surface. Subsequently, integrins on the leukocyte become activated and engage specific immunoglobulin-like counter-receptors on the endothelium, leading to the arrest of the leukocyte and extravasation into the tissue [5].

The selectins were named for the C-type lectin domains at their amino termini (Fig. 1) and their function in the recognition of specific cell-surface carbohydrates. The nature of the carbohydrates, and of the cell-surface macromolecules that bear them has been an area of intense scrutiny [1,6,7]. A major motivation is the development of selectin antagonists to prevent the abnormal entry of leukocytes into tissues during pathological inflammatory reactions [1,7]. In 1990–1991, a seminal advance was the discovery that the tetrasaccharide sialyl Lewis x (sLe^x; Fig. 2) is recognized by all three selectins. P-selectin and L-selectin also bind to a number of sulphated or phosphorylated compounds which bear no apparent relationship to sLe^x [6].

The selectins have distinct binding specificities for the relevant target cells, which has prompted the search for specific glycoprotein ligands [2,7]. As summarized in

Figure 1

The domain organization of the three selectins and their physiological ligands. PSGL-1 is the dominant neutrophil ligand for P-selectin; it is also present on other leukocytes. Some of the *O*-linked oligosaccharides contain sialyl $Lewis^x$-related structures. The amino-terminal region bears three potential sites for tyrosine sulphation, at least one of which is used. GlyCAM-1, an L-selectin ligand, is secreted and may perform signaling functions in the recruitment of lymphocytes to lymph nodes. It contains sulphated, sialylated and fucosylated oligosaccharides (see Fig. 2). The carbohydrate structures of CD34 and a subset of MAdCAM-1 are thought to be similar to those on GlyCAM-1. The E-selectin ligand, ESL-1 is predicted to bear up to five *N*-linked oligosaccharides, at least some of which are sialylated and fucosylated. Not shown in the figure are several ligands which have been described at the biochemical level [1,2]: Sgp200 and heparan sulphate proteoglycans for L-selectin; a 160 kD ligand for P-selectin; and ~ 200 kD (CLA) and 250 kD ligands for E-selectin.

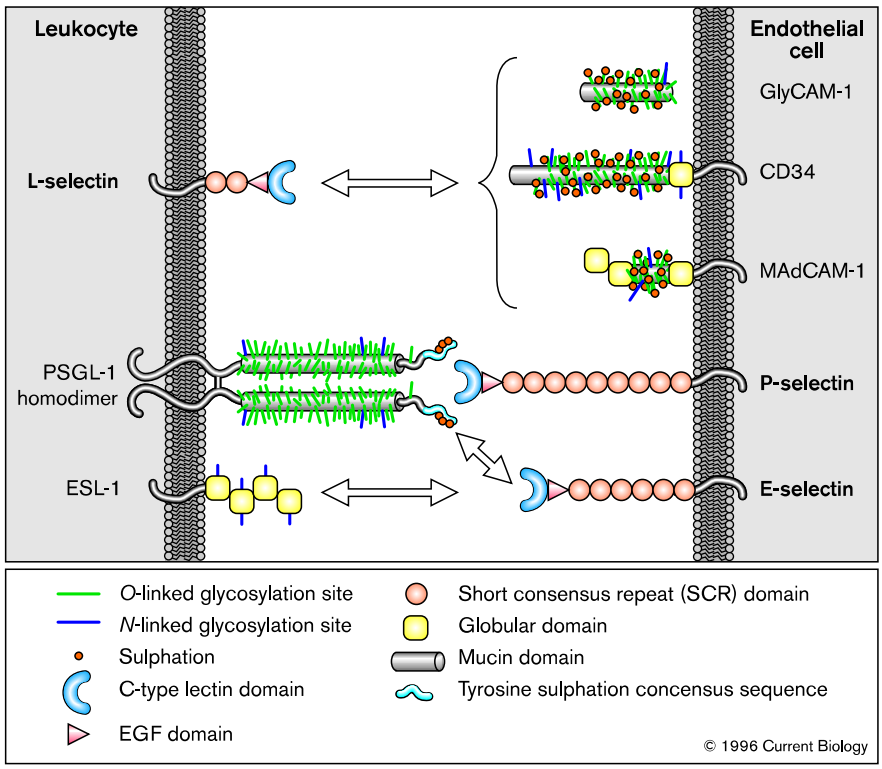


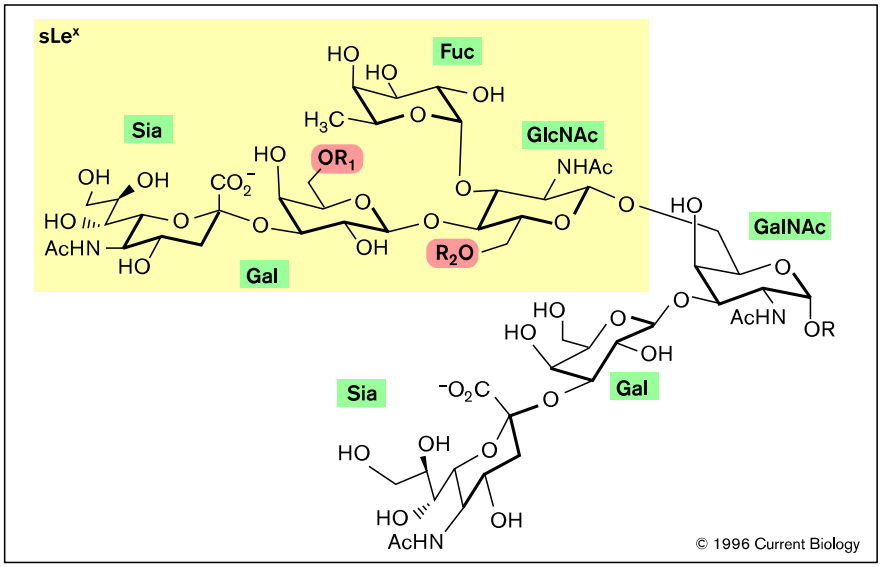
Figure 1, a number of ligands have now been identified. The best characterized of these is PSGL-1, which mediates P-selectin-dependent adhesive interactions under both static and flow conditions [2]. In the case of L-selectin, the situation is more complex, in that multiple ligands have been identified [1,4,6], including GlyCAM-1, CD34, Sgp200, MAdCAM-1 — all associated with high endothelial venules (HEVs) in mouse lymphoid organs — as well as heparan sulphate proteoglycans from cultured bovine endothelial cells. The functions of this constellation of molecules in L-selectin-dependent recruitment to lymphoid organs and inflammatory sites are undoubtedly complex and remain to be elucidated. For E-selectin, several myeloid ligands have also been identified. One of these is PSGL-1, although E-selectin appears to utilize distinct regions on PSGL-1, in addition to those recognized by P-selectin [2]. Notably, several of the selectin ligands (GlyCAM-1, CD34, MAdCAM-1 and PSGL-1), but not all (for example, ESL-1), contain mucin-like domains.

An extensive series of experiments identified sLe^x or related structures within PSGL-1, ESL-1, and the HEV-associated ligands for L-selectin [6]. The question of how the selectins achieve high affinity (10^4–10^5-fold greater than to sLe^x) and selectivity *in vivo* has aroused tremendous interest. A possible contributing factor to avidity could be the multivalent presentation of sLe^x-like determinants on mucin domains of the ligands. Affinity, as well as selectivity, could also derive from distinctive features of the carbohydrate chains or the protein core. As reviewed below, growing evidence indicates that sulphation is a critical feature for two classes of selectin ligands.

Sulphation of the principle HEV-associated ligands for L-selectin (GlyCAM-1, CD34 and Sgp200) is well established [1]. GlyCAM-1, the only ligand subjected to a detailed biochemical analysis, shows intense metabolic incorporation of sulphate into its *O*-glycans. Studies using chlorate — a selective inhibitor of 3'-phosphoadenosine 5' phosphosulphate synthesis (PAPS) — establish that sulphation is essential for the binding of the HEV ligands to L-selectin and to MECA 79, a function-blocking monoclonal antibody [8]. Analysis of GlyCAM-1 has shown that its *O*-glycans are extensively sulphated, with approximately equal contributions from Gal-6-sulphate and GlcNAc-6-sulphate (see Fig. 2 legend for abbreviations). The *O*-glycans comprise a complex array of chains of varying length and degrees of sulphation, and only the simplest of these structures has been deduced (Fig. 2) [9]. Notably, sLe^x capping groups modified with sulphate esters on the 6 position of Gal or the 6 position of GlcNAc are predicted to be major components of the GlyCAM-1 oligosaccharides.

On the basis of these structures, a number of simple sulphated derivatives have now been synthesized and tested for L-selectin binding. Sulphation of sLe^x on the 6-position of GlcNAc increases its L-selectin-binding potency four-fold in an equilibrium competition assay [10]. Furthermore, a lactose derivative substituted on the 6′ position of Gal (Fig. 2) shows significant L-selectin-binding activity and is only two-fold less potent than sLe^x [11]. Significantly, a disulphated version of lactose, containing sulphates on the 6 positions of both Gal and Glc, is actually two-fold more potent than sLe^x. These findings suggest that both types of sulphate modifications found within GlyCAM-1 contribute to L-selectin binding. The

Figure 2



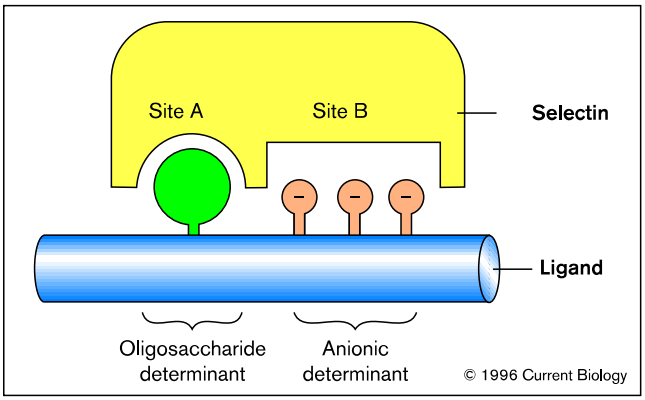
Predicted structures of the simplest *O*-glycans from GlyCAM-1, as deduced by metabolic radiolabeling studies. The oligosaccharides bear the sLe^x tetrasaccharide (highlighted) and are sulphated on the 6 position of Gal (R_1) and/or the 6 position of GlcNAc (R_2). Abbreviations: Sia, sialic acid; Gal, galactose; GlcNAc, *N*-acetylglucosamine; Fuc, fucose; GalNAc, *N*-acetylgalactosamine.

relative importance of each modification and the nature of the complete recognition determinants are unknown at present. In addition, the contribution of these sulphate esters to the kinetic parameters of ligand interaction has not, as yet, been determined.

In the case of P-selectin, early experiments with selenate (a metabolic inhibitor of sulphation) suggested the potential importance of sulphation for neutrophil ligands [12]. The molecular cloning of PSGL-1 reawakened interest in this question, with the finding that three consensus sites for tyrosine sulphation lie within the amino-terminal 10 amino acids of the mature protein [13]. In contrast, GlyCAM-1, CD34, and MAdCAM-1 do not have any such sites. Analysis of PSGL-1 derived from a neutrophil-like cell line (HL60) confirmed that PSGL-1 is sulphated and that all of the sulphate is bound to tyrosine residues [14]. Either removal of sulphate from PSGL-1 by an arylsulphatase, or cleavage of the first 10 amino acids from the ligand by a cobra venom metalloproteinase, results in a parallel loss of binding to P-selectin [14,15]. Strikingly, the amino-terminal region bearing the sulphated tyrosines is not required for E-selectin binding.

Recombinant functional PSGL-1 can be produced in COS cells by cotransfection of a cDNA encoding the core protein and a cDNA for a fucosyltransferase, which can give rise to sLe^x determinants [13]. A chimeric PSGL-1 linked to the Fc region of an antibody retains binding activity for P-selectin, even when it contains only the first 19 residues of PSGL-1 [16]. This minimal fragment contains the three potential tyrosine sulphation sites as well as a single site that is used for *O*-glycosylation. The binding of the truncated molecule depends on its fucosylation and sialylation, as does the binding of the parent PSGL-1 molecule. Mutation of either the tyrosines or the glycosylation site dramatically reduces binding to P-selectin. In striking contrast, tyrosine sulphation is not necessary for binding of this minimal chimera or larger fragments to E-selectin, although the same carbohydrate requirements apply. A complementary study showed that appending the amino-terminal region (20 residues) of PSGL-1 to the mucin-like stalk of CD43 confers P-selectin-binding activity [17]. Again, optimal ligand activity requires the critical tyrosines in the terminal segment and the appropriate carbohydrate modifications. Taken together, this remarkable series of studies leads to a two-site model for P-selectin binding to PSGL-1, in which there is obligate dual recognition of an anionic amino-terminal sulphated region and a sialylated/fucosylated determinant (Fig. 3). By contrast, recognition of PSGL-1 by E-selectin occurs independently of the amino-terminal region. Thus, the selective use of tyrosine sulphation provides one basis for the differential recognition of PSGL-1 by the two endothelial selectins.

Figure 3



The two-site model for binding of P- and L-selectins to their sulphated physiological ligands. Site A on the selectin recognizes an oligosaccharide determinant; site B recognizes an anionic determinant on the cognate ligand. In GlyCAM-1, the oligosaccharides are sulphated, sialylated and fucosylated; in PSGL-1 the oligosaccharides are sialylated and fucosylated, but unsulphated. Site A in P-selectin recognizes sLe^x-like oligosaccharides, while site B interacts with an anionic amino-terminal region containing tyrosine sulphate. Site A lies within the C-type lectin domain of P-selectin, whereas site B may be in either the lectin domain or the EGF domain. The distinction between sites A and B in L-selectin is less clear.

Carbohydrate recognition undoubtedly resides within the lectin domain of P-selectin, and some of the critical residues underlying the binding of sLe^x have been defined [4]. The site for recognition of tyrosine sulphates may also reside within the lectin domain, but the neighboring EGF-like domain must be considered as well (Fig. 1). The EGF domain is highly conserved across species, is required for functional binding to ligands, and appears to contribute to the specificity of P-selectin binding to myeloid ligands [3]. These observations are consistent with the direct participation of the EGF domain in recognition of the ligand (the anionic amino-terminal peptide), but a structural support role cannot be ruled out. The existence of a secondary binding site could explain why so many anionic substances with no apparent structural relationship to sLe^x bind to P-selectin [6].

The nature of L-selectin binding to its sulphated physiological ligands is more difficult to understand. In one model, sulphation of the oligosaccharides on L-selectin ligands may promote their interaction within the lectin domain. Alternatively, a secondary site may be involved in the recognition of these sulphated structures, while a primary lectin-domain site interacts with sLe^x-like oligosaccharides irrespective of sulphation. This second model is similar to a previous one in which combinations of oligosaccharides define the recognition structure [6]. The first model is supported by the observation that the relative L-selectin-binding activity of sulphated oligosaccharides is dependent on the positions of sulphation (see above). In considering the second model, it is noteworthy

that the binding of L-selectin to the anionic phospholipid cardiolipin has been localized to a positively charged region within the lectin domain (residues 84–89, Lys–Lys–Asn–Lys–Glu–Asp in human) [18]. This six-residue sequence, which lies in close proximity to residues involved in sLe^x binding [4], may comprise a secondary binding site for anionic substances. In E-selectin, this region has a net negative charge, which may account for the observation that E-selectin does not recognize the highly anionic substances that bind to P-and L-selectins.

The sites of attachment of sulphate esters to PSGL-1 and GlyCAM-1 are different (protein *versus* carbohydrate), yet both ligands have evolved a dependency on sulphation. What are the special properties of sulphate that have led to such a dramatic convergence of function? Sulphate esters are fully ionized at physiological pH and augment negative charge. Both L- and P-selectins have a large number of basic amino acids in their lectin domains, suggesting the possibility of favorable electrostatic interactions with anionic sites on cognate ligands. Thus, electrostatic attraction may explain the sulphation-dependent binding of PSGL-1 and GlyCAM-1 to P-selectin and L-selectin, respectively.

Sulphate recognition may have an important impact on the association rate of selectin–ligand binding. The selectins must support leukocyte rolling under conditions of rapid flow in the blood stream and high 'on rates' are believed to be essential for this function [5]. Interestingly, a relationship between electrostatic attraction and rapid association rates has been previously documented. Many DNA-binding proteins have regions of basic amino acids that interact non-specifically with the anionic phosphodiester backbone, resulting in an enhancement in association rate by electrostatic attraction [19]. In fact, attachment of basic peptides to a single-stranded oligonucleotide can lead to dramatic increases in the rate of association (48 000-fold) with a complementary nucleotide strand [20]. An adhesion-related example is seen in the interaction of the platelet GP Ib–IX complex with von Willebrand factor (vWF) [21]. This shear-induced binding event initiates the attachment of platelets to the blood vessel wall at sites of injury, an event that must occur very rapidly. Tyrosine sulphation within GP Ibα, a component of the complex, is essential for vWF binding. Thus, it is striking that several molecules involved in rapidly occurring adhesive events within the blood vascular compartment share a dependency on sulphation. The possible kinetic advantage conferred by sulphate may explain the convergence of two selectins on this modification.

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